Original Article

Pollen-pistil interaction and early fruiting in parthenocarpic citrus

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Background and Aims An intense pollen-pistil interaction precedes fertilization. This interaction is of particular relevance in agronomic important species where seeds or fruits are the edible part. Over time some agronomic species have been selected for the ability for to produce fruit without seeds. While this phenomenon is critical for commercial production in some species, very little is known about the events behind the production of seedless fruit. In this work we investigate the relationship between pollen-pistil interaction and the onset of fruiting in Citrus mandarin.

Methods Pistils were sequentially examined in hand pollinated flowers paying attention to pollen tube behaviour, and to cytochemical changes along the pollen tube pathway. To evaluate what of these changes were induced by pollination/fertilization and which were developmentally regulated, pollinated and unpollinated pistils were compared. Also the onset of fruiting was timed and changes in the ovary examined.

Key Results Conspicuous changes occurred in the pistil along the pollen tube pathway, which took place in a basipetal way encompassing the timing of pollen tube growth. However, these changes appear to be developmentally regulated for they happened in the same way and at the same time in unpollinated flowers. Moreover, the onset of fruiting occurred prior to fertilization and the very same changes could be observed in unpollinated flowers.

Conclusions Pollen-pistil interaction in Citrus showed similarities with unrelated species and families belonging to other taxa. The uncoupling of the reproductive and fruiting processes accounts for the parthenocarpic ability of unpollinated flowers to produce fruit in Citrus. However, the maintenance of a functional reproductive process reflects the potential to produce seeded fruits, providing a basis for the understanding of the production of seeded or unseeded fruits and further understanding of the process of parthenocarpy in other species.

Key words: *Citrus*, flower development, mandarin, obturator, ovary, papillae, parthenocarpy, pistil, pollen tube competition, seedlessness, stigma, stylar canal.

INTRODUCTION

Pollen-pistil interaction precedes fertilization in the flower. Important changes occur in the pistil, which play a role supporting, but also controll pollen tube growth (Herrero and Hormaza, 1996; Hiscock and Allen, 2008; Kumar and McClure, 2010). One of the best known control systems of pollen-pistil interaction is pollen-pistil incompatibility (Heslop-Harrison, 2000; McClure and Franklin-Tong, 2010), but pollen-pistil interaction also play an important role in pollen competition and selection during compatible mating (Hormaza and Herrero, 1992; Kumar and McClure, 2010). Other changes do play a preventative role in what could be a vulnerable entrance point for pathogens (Heslop-Harrison, 2000). Changes in the pistil also regulate pollen tube directionality (Herrero, 2000, 2003; Stewman *et al*., 2010), and the localization of mutants in model species is showing the genes responsible for these interactions (Chapman and Goring, 2010, Lausser *et al*., 2010). Failure in these genes results in failure in seed production.

Pollen-pistil interaction has a particular relevance in agronomic important species were seed of fruit production are the edible part. However, agriculture practice along the years appears to have selected in some species the production of fruits without seeds (Varoquaux *et al*., 2000), as it occurs in bananas (Heslop-Harrrison and Schwarzacher, 2007) and citrus (Talon *et al*., 1992). This trait may be desirable in species were seeds are a nuisance at eating and has also recently being induced in other species as watermelon or grapes (Bouquet and Danglot, 1996; Sugiyama *et al*., 2002); or found in a natural mutant of Annona (Lora *et al*., 2011). Agricultural selection has surely being towards failures along the reproductive process. However, very little is known on the mechanisms behind this lack of seeds in traditionally seedless crops. Apart from the fact that parthenocarpic

fruit development is triggered by a deregulation of the hormonal balance (Vivian-Smith *et al*., 2001; Gorguet *et al*., 2005). In particular, altered levels of phytohormones have been observed during fruit growth in naturally occurring parthenocarpy in citrus plants (Talon *et al*., 1990, 1992). Studies in mutants of tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana* have revealed that the hormonal signaling pathway is implicated in repressing fruit initiation in the absence of the fertilization resulting in parthenocarpic fruit (Vivian-Smith *et al*., 2001 Goetz *et al*., 2007). A different mechanism has recently been reported in Annona, where deletion of the INO locus, responsible for ovule outer integument development, results in seedless fruits in a natural mutant (Lora *et al*., 2011). Citrus appear as especially interesting, because they are often seedless, but also, at least some of them as mandarins, maintain their capacity to produce seeds.

Citrus species are grown in tropical and sub-tropical climates, being the base of a prosperous industry in more than 100 countries, with a total annual production over 120 million tones (FAOstat, 2009). Citrus are an old crop, been cultivated for over 4000 years, with a great diversity and apparently distant centers of origin (Khan, 2007). This variability contrasts with the permissive intercrossability between species. Indeed, the vast majority of cultivated citrus are derived from interspecific crosses between ancestral species (Nicolosi *et al*., 2000). In spite of this variability, a vast majority of these species are parthenocarpic and able to produce seedless fruits without fertilization. This character could have been early selected and be related to their early domestication and growing.

In mandarins not all cultivars are equally parthenocarpic. However, the recent introduction of new mandarin cultivars to widen the production calendar has resulted in the production of seeded fruits. This occurrence is a problem that causes serious economic losses in different parts of the world, and a number of alternatives have been explored (Vardi *et al*., 2008), which range from the regulation of pollen flow (Chao *et al*., 2005) to

the use of treatments as insect repellents (Pons *et al*., 1996), or to reduce pollen viability (Mesejo *et al*., 2006). In addition, a number of breeding strategies have been explored such as the introduction of cytoplasmic male sterility (Guo *et al*., 2004), ploidy manipulation for increasing sterility (Grosser *et al*., 2000; Grosser and Gmitter, 2005; Navarro *et al*., 2004; Reforgiato *et al*., 2005), induced mutation (Spiegel-Roy and Vardi, 1981) and transgenic approaches (Li *et al*., 2002). However, a definitive answer is still elusive. The search for alternatives contrasts with the paucity of data on the underlying cause of the problems, namely the reproductive process.

Indeed, information on the processes underpinning fruit production in Citrus, which constitutes the basis of this industry, is surprisingly thin. However, embryologists were interested in Citrus early. Braun (1860) and Strasburger (1878) recorded the occurrence of polyembryony, and this research was followed by the more detailed work of Osawa (1912). Flower-bud differentiation was described long ago (Abbot, 1935) as well as the flower anatomy of the Aurantiodeae (Tillson and Bamford, 1938) and lemon (Ford, 1942). Later, the ultrastructure of stigma and style of *C. limon* was characterized (Ciampolini *et al*., 1981; Cresti *et al*., 1982) as well as the general structure of the gynoecium (Soost and Roose, 1996). Additionally, pollen tube behavior was recorded in compatible and incompatible pollinations (Ton and Krezdorn, 1966; and Kahn and De Mason 1986, 1988). These studies reveal a well conserved anatomy of the gynoecium in different species of *Citrus*. However, information is lacking on the reproductive process and the early changes accompanying the onset of fruiting.

In this work, we examined the pollen-pistil interaction in a widely grown mandarin 'Nova' [tangelo Orlando (*C. paradisi* Macf. x *C. reticulata* Blanco) x *C. clementina* Hort *ex* Tan] and relate the sequence of events with those occurring at the onset of fruiting. The sequential comparison, of pollinated and unpollinated flowers, sheds light on which of

these processes are induced by pollination/fertilization and which are developmentally regulated, as well as providing a reference line for understanding the fruiting process in this genus.

MATERIALS AND METHODS

Plant material

Adult trees of the 'Nova', and 'Fortune' (*C. clementina* Hort ex Tan. x *C. reticulata* Blanco) cultivars grown at the "Primosole" experimental farm of University of Catania in Sicily were used for this study. These were chosen for their wide diffusion in major citrus-producing Mediterranean countries.

Pollination procedures

Pollen was obtained from 'Fortune'. For this purpose, anthers from flowers were collected one day prior to anthesis. The anthers were left to dehisce on paper at room temperature (about 25 °C) for one day, and fresh pollen was immediately used for pollination. Additionally, 'Nova' flowers, were emasculated one day before anthesis, pollinated with pollen from 'Fortune,' and bagged with cotton tissue. A batch of pistils, similarly emasculated and bagged, was left unpollinated.

Microscopy

Pollinated and unpollinated pistils of 'Nova' were sampled sequentially, every two days for 7 times, until 14 days after pollination. The collected pistils were cut into three sections (stigma, style and ovary). Ten pistils per treatment were fixed in 2·5 % glutaraldehyde in 50 mM phosphate buffer (pH 7·2), dehydrated in a graded ethanol series up to 70 % ethanol, and stored at 4 °C. Subsequently, they were dehydrated in 100 %

ethanol, embedded in JB-4 methacrylate (Polysciences Co. Ltd, Eppelheim Germany), and sectioned at 3 μm on a rotary microtome (Leica, Wetzlar, Germany). Ten additional pistils were sampled and treated each day. These samples were fixed in FAA solution (5 mL formalin / 5 mL glacial acetic acid / 90 mL 70 % ethanol, v/v/v; Johansen 1940), washed in 50 % ethanol for an hour, and stored at 4 °C. Subsequently, they were dehydrated and transferred to paraffin using TBA (tertiary butyl alcohol) as the intermediate solvent. The samples were left in liquid paraffin at 60 °C for 3 weeks and embedded.

Parafin sections, 10 μm thick, were stained with 0·1 % aniline blue in 0·1 N K_3PO_4 (Linskens and Esser, 1957) to observe pollen tubes. Additionally, starch was localized with 2 % IKI (Johansen, 1940). JB-4 sections were stained with periodic acid – Schiff's reagent (PAS) (Jensen, 1962) for carbohydrates, 0·07 % calcofluor (Hughes and McCully, 1975) for cellulose, 0·01 % auramine (Heslop-Harrison, 1977) for cutin, 0·01 % acridine orange (Fleming *et al*., 1993) for RNA, and 0·02 % tolouidine blue (O'Brien *et al*., 1964) for general staining.

Sections stained with PAS, tolouidine blue, and IKI were observed with bright field microscopy. Sections stained with aniline blue, calcoflour, auramine, and acridine orange were observed using a fluorescence microscope (Leica DM 2500 of Leica, Wetzlar, Germany) using I3 filter excitation 450-490 nm, for aniline blue and calcoflour, N2·1 filter (515–560 nm) for auramine, and A filter (340–380 nm) for acridine orange.

Evaluation of ovary growth

To evaluate the onset of fruiting, 10 flowers/fruitlets, were individually weighed every five days from anthesis to 20 days later. This procedure was performed in pollinated and unpollinated flowers.

RESULTS

Pistil anatomy

The gynoecium was formed from 10 to 11 carpels fused together, which gathered around an inner channel. While they all shared a continuous common stigma (Fig. 1), each carpel has an independent stylar canal leading to each locule, which hosted 3 to 4 ovules.

The stigma surface had unicellular and multicellular papillae (Fig. 1A), which varied in size and averaged 64·6±18·9 μm. The papillae showed an increase in length toward the upper part of the stigma, and they were smaller in the stigmatic stylar canal openings. Small amyloplasts were observed in the papillae at the flower opening. Later in development, the papillae became highly vacuolated cells and produced an exudate, which covered the stigmatic surface (Fig. 1B) and were stained by auramine, acridine orange and PAS.

Stylar canals were layered radially in a common cylindrical style that had a central hollow channel in the middle (Fig. 1C). Each stylar canal looked like a buttonhole bordered by papillar cells. The stylar canal cells had a rich cytoplasm, and the cell walls facing the stylar canal were thick and secretory, with a secretion filling this canal (Fig. 1D).

Stylar canals descend towards the ovary and lead into the ovary locule, at the inner angle of each locule (Fig. 1E), in an area continuous with the stylar canal papillae that is covered by long starch-rich papilla (Fig. 1F). The placenta bears three to four anatropous ovules per locule. The ovule exostome, formed by the opening of the outer integument of the ovule, was also rich in amyloplasts and faced the papillae. However, at anthesis, these papillae did not reach the ovule exostome, and left a gap between them.

Pollen tube growth

Pollen grains germinated on the stigmatic surface (Fig. 2A) and the pollen tubes reached the upper part of the style 48 hours after pollination. Once germination had occurred, pollen grains appeared empty of their cytoplasmic content (Fig. 2B). The stylar canals appeared on the surface of the stigma and pollen tubes grew towards them and along the stylar canal close to the papillae bordering cells. The first pollen tubes reached the base of the style some 6 days after pollination.

About the 75 % of pollen grains germinated on the stigmatic surface and over 100 pollen tubes reached the upper part of style. This number decreased as the pollen tubes grew along the stylar canals, and only some 8 to 12 pollen tubes got down to the base of the style. This reduction was accompanied by a reduction in the size of stylar canals (Figs. 2C, 2D, 2E), which were reduced in surface area from some 277 μm to 142 μm, as they descended towards the ovary. This reduction was due to a decrease in the number of cells bordering the stylar canal (Table 1). Pollen tubes reached the ovary locule through the long papillar hairs, which were continuous with the stylar canals (Fig. 2F). The pollen tube penetrated into the micropyle (Fig. 2G) and traversed the nucellus. This event occurred 12 days after pollination. Sometimes it was possible to see pollen tubes growing out of the stylar canals within the central channel of the style.

Changes in the stigma-style

Conspicuous changes occurred in the stigma-style after flower opening. These changes occurred along the internal space of the style and also along the pollen tube pathway in the stigma and the style.

At anthesis, the internal channel, around which the different carpels gathered, appeared as an empty space (Fig. 3A) bordered by cells with thick walls (Fig. 3B). These

cells appeared the same along the length of the channel. However, later, these cells changed their appearance and entered a secretory phase, which involved the production of a secretion that filled this area (Figs. 3C and 3D). The secretion was basipetally produced, which started at the upper part of style, continued through the style, and all the way down to the ovary. Secretory activity began 4 days after anthesis in the upper part and filled the entire hollow inner space 6 days later. Both the walls of these cells and the secretion reacted positively to staining with auramine, acridine orange and PAS. These changes occurred in the same way and at the same times in pollinated and unpollinated flowers.

Clear changes also occurred along the pollen tube pathway. The stigmatic papillae had starch at anthesis although variability in the amount of starch between the different flowers of a same tree could be observed. Smaller amyloplasts of a different color (blue, red, orange, brown) than in parenchymatic cells were observed in papillae (Fig. 4A). However, shortly after flower opening, starch disappeared as the stigmatic secretion increased. The papillae vacuolated and secretion could be observed mainly in the papillar tips (Fig. 4B). This event occurred both in pollinated and unpollinated flowers. However, in pollinated stigmas, stigmatic secretion had a looser appearance (Fig. 4C) than in unpollinated stigmas, which appeared denser.

Cells bordering the stylar canal also had small starch grains at anthesis (Fig. 4D). During pollen tube growth, starch reserves in the stylar canal cells were degraded as a secretion appeared (Fig. 4E), to fill the internal canal (Fig. 4F). In unpollinated flowers, the stylar canal cells also entered a secretory phase, but kept some of their starch reserves (Fig. 4G). This occurred at the same time than in pollinated flowers. But the appearance of the style was completely different in pollinated flowers following pollen tube passage. In these styles, the cells were depleted of starch and the secretion had also vanished, while profiles of pollen tubes occupied the secretion area (Fig. 4H).

Changes in the ovary

In the ovary, the papillar cells in the placenta, which are continuous with the stylar canal cells, also exhibited conspicuous changes. These papillar cells were short at anthesis (Fig. 5A), but grew and extended as the flower aged (Fig. 5B), approaching the ovule exostome. Ten to 12 days after anthesis, epidermal hairs became two-celled by a transverse division and appeared more than two fold longer than at anthesis. At anthesis, papillar cells had some starch and a well-defined cell wall (Fig. 5C). However, later on, the elongated papilla entered a secretory phase (Fig. 5D) and starch vanished in the most external part close to the pollen tubes. Secretions were stained with auramine, acridine orange and PAS. Following the production of the secretion, these cells became highly vacuolated. Secretion in this area started 6 days after anthesis and was most conspicuous ten days after anthesis. Pollen tubes were observed traversing this area 12 days after pollination. A sequential comparison of pollinated and unpollinated flowers showed that papillar cell elongation and the production of secretion occurred in the same way and at the same time in both pollinated and unpollinated flowers.

Conspicuous changes were also observed in the ovary locules. At anthesis, some protuberances appeared in the locule wall (Fig. 5E), which continued to develop into the juice vesicles. These changes were most conspicuous 10 days after pollination (Fig. 5F) and prior to fertilization and could also be observed at this time in unpollinated flowers.

Early fruit growth

To evaluate the onset of fruiting, 10 flowers/fruitlets were weighed every 5 days in both pollinated and unpollinated flowers. Clear differences existed between flowers/fruitlets within one sampling day, and these differences were most apparent in

most advances dates. Ovary growth could be observed some 5 days after anthesis and was clearly apparent 10 days after pollination (Fig. 6). This growth occurred prior to fertilization. Surprisingly, unpollinated flowers had a similar continuous growth that was similar in weight to that of pollinated flowers.

DISCUSSION

The anatomical features of the mandarin gynoecium are conserved with those described in other *Citrus* species. However, the developmental approach of this work reveals that the pistil exhibited conspicuous changes during the flower lifespan. These changes played a clear part supporting, but also constraining, pollen tube passage in both the style and also in the ovary. Most of them appeared to be developmentally regulated because they occurred the same way in unpollinated species. This observation also applied to the onset of fruiting, which occurred independently of fertilization.

A support-constrain strategy

The stigma of the 'Nova' mandarin was very similar to the stigma of lemon (Cresti *et al.,* 1982), with unicellular and multi-cellular papillae that are variable in size, and covered with a conspicuous secretion that plays a part in pollen capture, adhesion and germination as it occurs in other species (Dumas *et al*., 1984; Swanson *et al*., 2004, Hiscock and Allen, 2008). While stigmatic secretion was produced equally in pollinated and unpollinated stigmas, this secretion changed in appearance following pollen grain germination.

The stylar canals were continuous with the papillar surface of the stigma and were also filled with a secretion. However, this secretion was not present at the beginning of anthesis and was produced in a basipetal way starting underneath the stigma and

continuing down to the base of the style. Interestingly, the timing for secretion production was concomitant with pollen tube growth along these stylar parts. While no evident changes were observed in the parenchymatic cells of the style, small amyloplasts in the cells bordering the stylar canal cells completely disappeared during pollen tube growth, which has also been observed in species with a transmitting tissue (Herrero and Dickinson, 1979; Ciampolini *et al*., 1981). There is mounting evidence that pollen tube growth, in solid or hollow styles, is heterotrophic at the expanse of the stylar reserves (Herrero and Dickinson, 1981). In hollow styles, this extracellular secretion has been shown to incorporate into the growing pollen tubes (Kroh *et al*., 1971; Labarca and Loewus, 1973), and more recent evidence supports this observation in other species (McClure, 2009).

The pistil of mandarin appears to be a structure especially well adapted to support pollen tube growth as it occurs in other species (Herrero and Hormaza, 1996). However, this support apparently is not unrestricted, and a reduction in the number of pollen tubes growing along the style was observed. A reduction in the number of pollen tubes was previously reported in other *Citrus* species (Kahn and DeMason, 1986). Similarly, a reduction in the number of pollen tubes travelling along the style has been recorded in compatible pollinations in a number of unrelated species (Cruzan, 1996; Herrero and Hormaza, 1996), and it is especially noticeable in species where the number of germinating pollen grains is higher than the number of ovules available for fertilization (Hormaza and Herrero 1994). This pollen tube attrition has been proposed to constitute a substrate for pollen tube competition and prezygotic selection (Mulcahy, 1979; Hormaza and Herrero, 1994; Erbar, 2003).

The anatomy of the stylar canals, in mandarin, could support this strategy because they become smaller as they descend towards the ovary. This reduction in size has not been reported before, and it is due to a decrease in the number of cells that border the stylar

canal. A similar fact has been described in other species with a solid style. In *Prunus persica*, a reduction in stylar reserves appears to be caused by a progressive reduction in the physical space occupied by the stylar transmitting tissue and also by a reduction in the amount of carbohydrates stored in this region (Herrero, 1992). It has been proposed that these anatomical changes could play a role favoring pollen tube competition and thus facilitating pollen selection (Tilton *et al*., 1984; Hormaza and Herrero 1994), with possible evolutionary and adaptive implications (Hedhly *et al*., 2007, 2009).

Pollen tube growth in the ovary

In the ovary, conspicuous changes occurred during development. The papillae, which are continuous with the cells bordering the stylar canals, elongated reaching proportions two fold longer and thinner than at anthesis. This growth allowed them to approach the ovule exostome as they entered a secretory phase. Pollen tubes reached the ovules through these elongated papillar cells concomitantly with this secretory phase. Thus, these papillar hairs appeared to play a role similar to an obturator, a placental proturberance that becames secretory at a particular time (Arbeloa and Herrero, 1987), which then acts as a draw-bridge connecting the ovule with the base of the style.

Obturators have been observed in a number of unrelated species and families, such as Euphorbiaceae, Rosaceae, and Liliaceae, as well as in many other taxa. They also have different morphological structures, such as a pad or swelling, hairs, filaments or tufts (Tilton and Horner, 1980) and appear to establish contact between the ovule micropyle and the placenta (Endress and Matthews, 2006). A chemotropic and guidance function was proposed by Tilton et al. (1984), and in some species like *Prunus*, it has been shown that the obturator regulates pollen tube access to the ovule through the production of a secretion at a particular time (Arbeloa and Herrero, 1987). Likewise, the physical approaching of the

area of pollen tube growth towards the ovule also occurs in other species, like in *Pistacio*, with the development of a ponticulus (Martinez-Pallé and Herrero, 1995).

Although a function for the papillar hairs here described in *Citrus* has not been previously reported, the presence of papillar hairs in this area is ubiquitous in a wide range of *Citrus* species. These structures were first described in *C. limon* (Ford, 1942) and later in *C. grandis* (Banerji, 1954) and *Poncirus trifoliata* (Boeswinkel, 1978), and they can be multicelled as in *C. australasica* or unicelled as in *C. australis* (Clarke and Prakash, 2001). It might be worth determining whether such a similar function also occurs in other *Citrus* species.

A similar mechanism relating to the control of pollen tube entrance into the ovary was described in *Zea mays*, in which papillar hairs also cover the ovary entrance. As the pollen tubes pass by, these hairs loose turgidity once fertilization has occurred preventing other pollen tubes from entering this region (Heslop-Harrison *et al*., 1985). Intraovarian trichomes are taxonomically widespread in monocotyledons, but they are rare in dicotyledons. In contrast to dicotyledons, monocot intraovarian trichomes are always associated with mucilage secretion (Rudall *et al*., 1998). It might be worth determining whether or not they could also be involved in pollen tube passage and regulating pollen tube access to the ovule. Pollen tube guidance in the ovary is an active field where sporophytic (Chapman and Goring, 2010; Stewman *et al*., 2010) and gametophytic (Okuda *et al*., 2009, Yang *et al*., 2010) signals are supplied for pollen tube orientation. Results herein, provide a frame to elucidate the function of these signals. Likewise, multicellular stigmatic hairs with independent functions from stigma and style were studied for comparative studies of pollen-tube growth in early angiosperms (Prychid *et al*., 2011).

Developmental changes in unpollinated flowers

The pistil in mandarin appears well adapted to support pollen tube growth. A basipetal maturation starting at the stigma and continuing down to the ovary is carried out by a number of changes that occur at particular times, which support pollen germination and pollen tube growth along the style, but also pollen tube access to the ovule in the ovary. The fact that these changes are not dependent on the action of pollen tubes, but occur in the same places and at the same time in unpollinated flowers support the idea that these changes are developmentally regulated. Pistil development appears to be a continuous process throughout the flower lifespan and this has been described in other species (Herrero and Dickinson, 1980; Herrero and Gascón, 1987; Herrero and Arbeloa, 1989). Maturation occurred in a basipetal way, starting at the stigma and proceeding down to the ovary as is also the case in other species (Herrero and Arbeloa, 1989; Lora *et al*., 2010). Changes in the pistil appeared to occur during the growth of the pollen tube, or rather, pollen tube growth encompassed pistil development (Herrero and Arbeloa, 1989).

While a number of developmental changes in the pistil have also been found in other species, in most species, fruiting is a response to fertilization (Lee, 1988). Flowering plants usually require fertilization to form fruit and seed and to initiate floral organ abscission in structures that do not contribute to the fruit (Vivian-Smith *et al*., 2001). Fertilization of the ovule generally triggers the development of the ovary into a fruit (Gillaspy *et al*., 1993). Ovule abortion or failures along the reproductive process have been observed in mutants of *Arabidopsis* and tomato revealing that, while fruit initiation is normally inhibited in the absence of fertilization, alterations in the hormonal signaling results in parthenocarpic fruit (Vivian-Smith *et al*., 2001; Goetz *et al*., 2007).

However, the onset of fruiting in mandarin appeared to be independent of fertilization time because fruit growth started prior to fertilization. Additionally, the same observation was seen in the development of the juice vesicles. Surprisingly, these changes

also occurred in the same way and time in unpollinated flowers, which reflects an uncoupling of the fertilization and fruiting time processes. This uncoupling supports the parthenocarpic tendency long reported in mandarins and in most *Citrus* species (Talon *et al*., 1992) and these results help elucidate the parthenocarpic tendency in this genus. Finally, the approach of this work may help to elucidate the events behind parthenocarpy in agricultural important species.

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FIGURE LEGENDS

FIG. 1 Pistil anatomy of the 'Nova' mandarin. (A) A common stigmatic surface gives way to different stylar canals. (B) The stigma surface is covered by unicellular and multicellular papillae varying in size. (C) A cross section of a style with the stylar canals located radially and an inner channel (D) Each stylar canal is bordered by papillar cells. (E) Stylar canals come out in the ovary locule facing the ovules (arrows). (F) Papillar cells bordering on the stylar canals are continuous with the papilla in the placenta. JB-4 embedded longitudinal (A, B) and transversal (C, D, E, F) sections of the pistil stained with PAS and Toluidine blue. Scale bar = 50 μm.

FIG. 2 Pollen tube growth, in 'Nova' mandarin. (A) Germinating pollen grains in the stigma with pollen tubes oriented toward the stylar canals opening (white line) on the surface of the stigma. (B) Germinated pollen grains (PG) with pollen tubes growing

between the stigmatic papillae. (C-E) Pollen tube growth in the style in the upper (C), middle (D), and lower (E) part, showing a reduction in pollen tube number (arrows) along the style, which is accompanied by a reduction in surface area and in the number of cells bordering the stylar canal. (F) Pollen tubes (arrow) reaching the ovary growing on the long papillae, and (G) penetrating the ovule through the micropyle. Paraffin embedded longitudinal (A, B) and transversal (C, D, E, F, G) sections of the pistil stained with Aniline blue. Scale bar = 50 μm.

FIG. 3 Central channel modification during flower maturation in 'Nova' mandarin. (A) At anthesis, in the central channel, an empty space can be observed, (B) which is bordered by cells with thick walls. Some of the cells in A and B show stained lipidic contains (C) Four days after flower-opening cells bordering the central channel changed their appearance and entered a secretory phase (D) as the cells became papillated. JB-4 embedded transversal sections of the central canal stained with Auramine. Scale bar = 50 μm.

FIG. 4 Changes in the stigma and style in 'Nova' mandarin. (A) Stigmatic papillae at anthesis with small starch grains (black arrows) with a different appearance than starch in parenchymatic cells (white arrows). (B) Stigmatic papillae 2 days after anthesis covered with a stigmatic compact secretion (black arrow). (C) Germinated pollen grain (PG) in degraded stigmatic secretion in a flower 2 days after pollination. (D) Cells bordering the stylar canal with starch at anthesis. (E, F) Progressive degradation of starch reserves in the cells and secretion in the stylar canal 2 and 4 days after anthesis. (G) Stylar canal of an unpollinated flower 6 days after anthesis with a conspicuous secretion and still small starch grains (black arrows). (H) Stylar canal of pollinated flower 6 days after pollination, without starch grains, and the pollen tubes (white arrows) growing in the stylar canal. JB-4

embedded longitudinal (A, B, C) and transversal (D, E, F, G, H) sections of the stigma and style stained with PAS. Scale bar = 50 μm.

FIG. 5 Changes in the ovary in 'Nova' mandarin. Comparison of the ovary at anthesis (A, C, E), and 10 days later (B, D, F). (A) Stylar canal lined with papillar cells come out into the ovary locule. (B) Papillae have elongated 10 days after anthesis and are approaching the ovule. (C) Short papillae at anthesis with starch grains. (D) Elongated papillar cells with secretion. (E) Ovary locule at anthesis with small protuberances. (F) Development of juice vesicles 10 days after anthesis. JB-4 embedded transversal sections of the ovary stained with PAS. Scale bar = 50 μm.

FIG. 6 Onset of fruiting. Weight of flowers/fruitlets, from anthesis to 20 days later, in pollinated and in unpollinated flowers. Unpollinated flowers had a similar continuous growth that paralleled in weight to that of pollinated flowers.

TABLE 1. *Radial length and number of cells on one side of the stylar canals at different levels of the style.*

	Upper style	Middle style	Lower style
Radial length (μm)	277±35	193±14	143±15
Number of cells	57±5	37±4	28±3

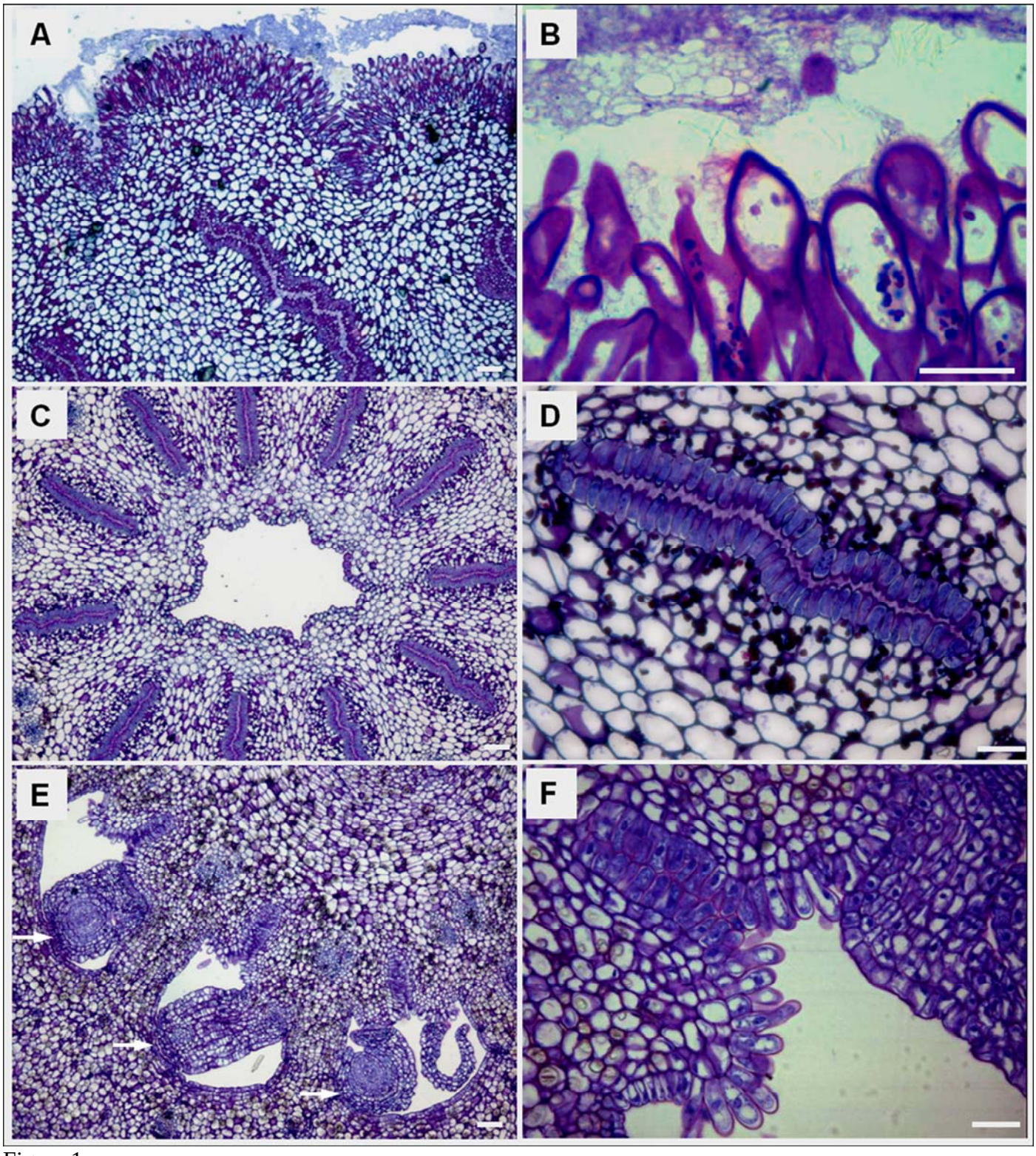

Figure 1

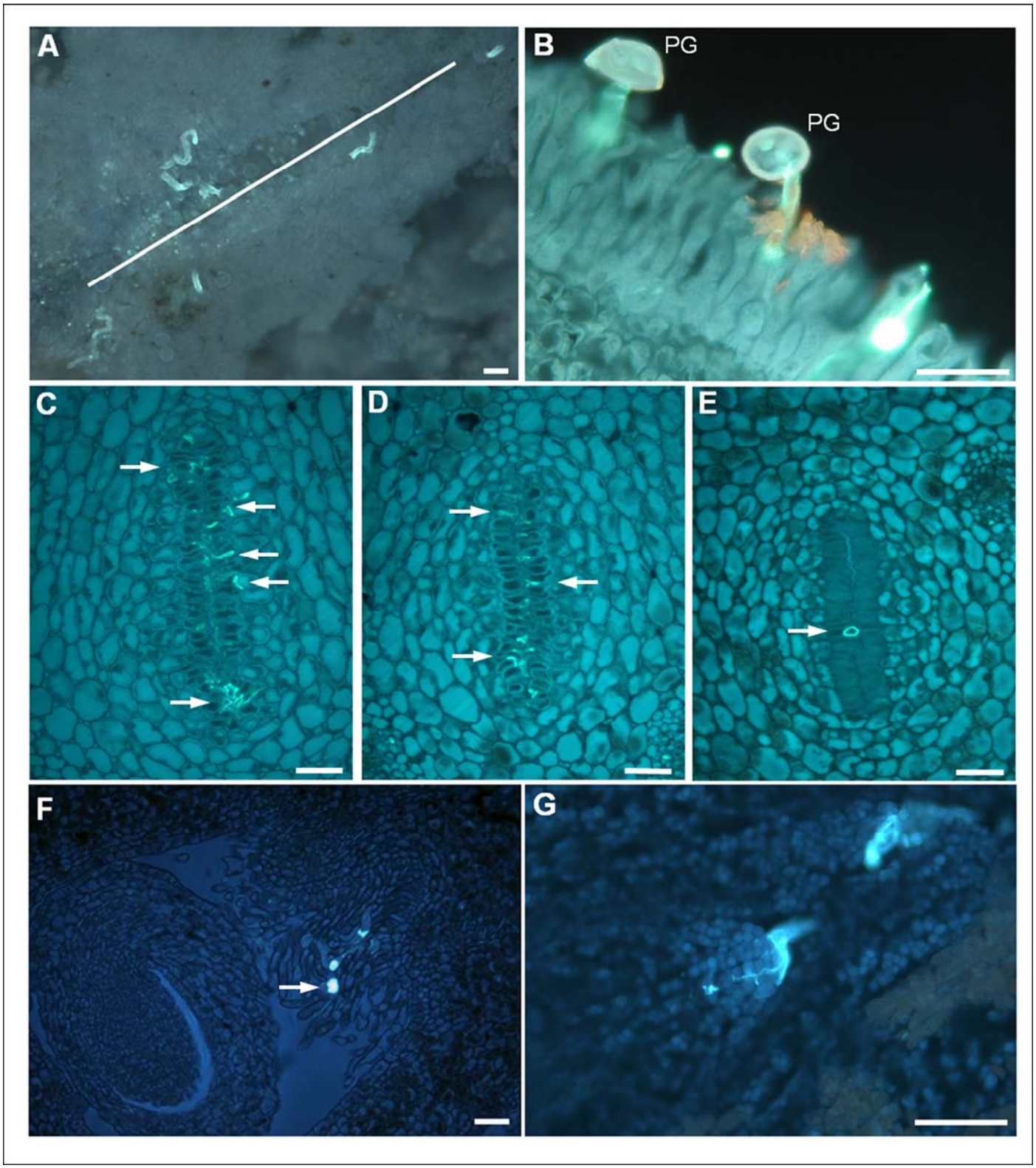

Fig. 2

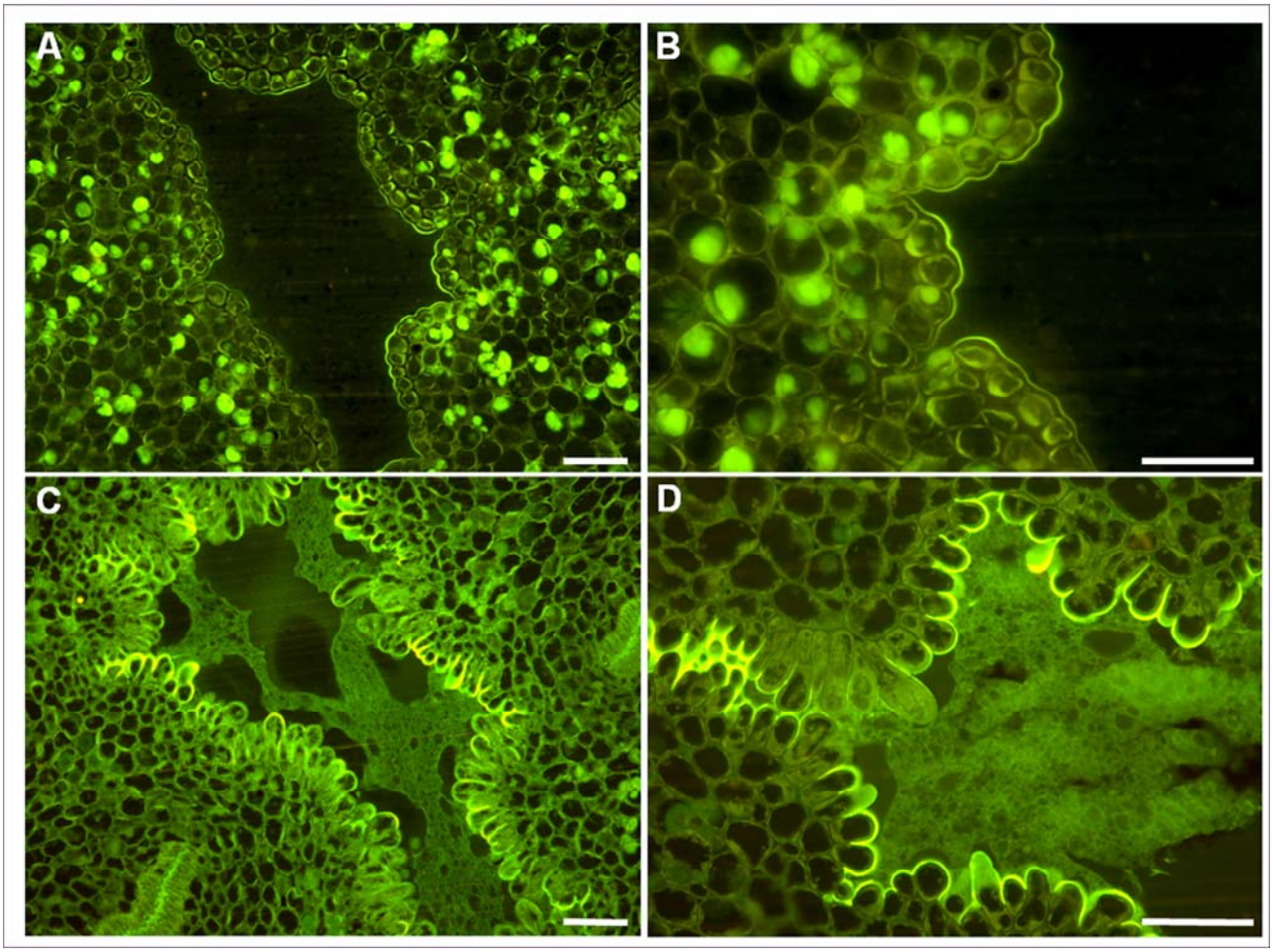

Fig. 3

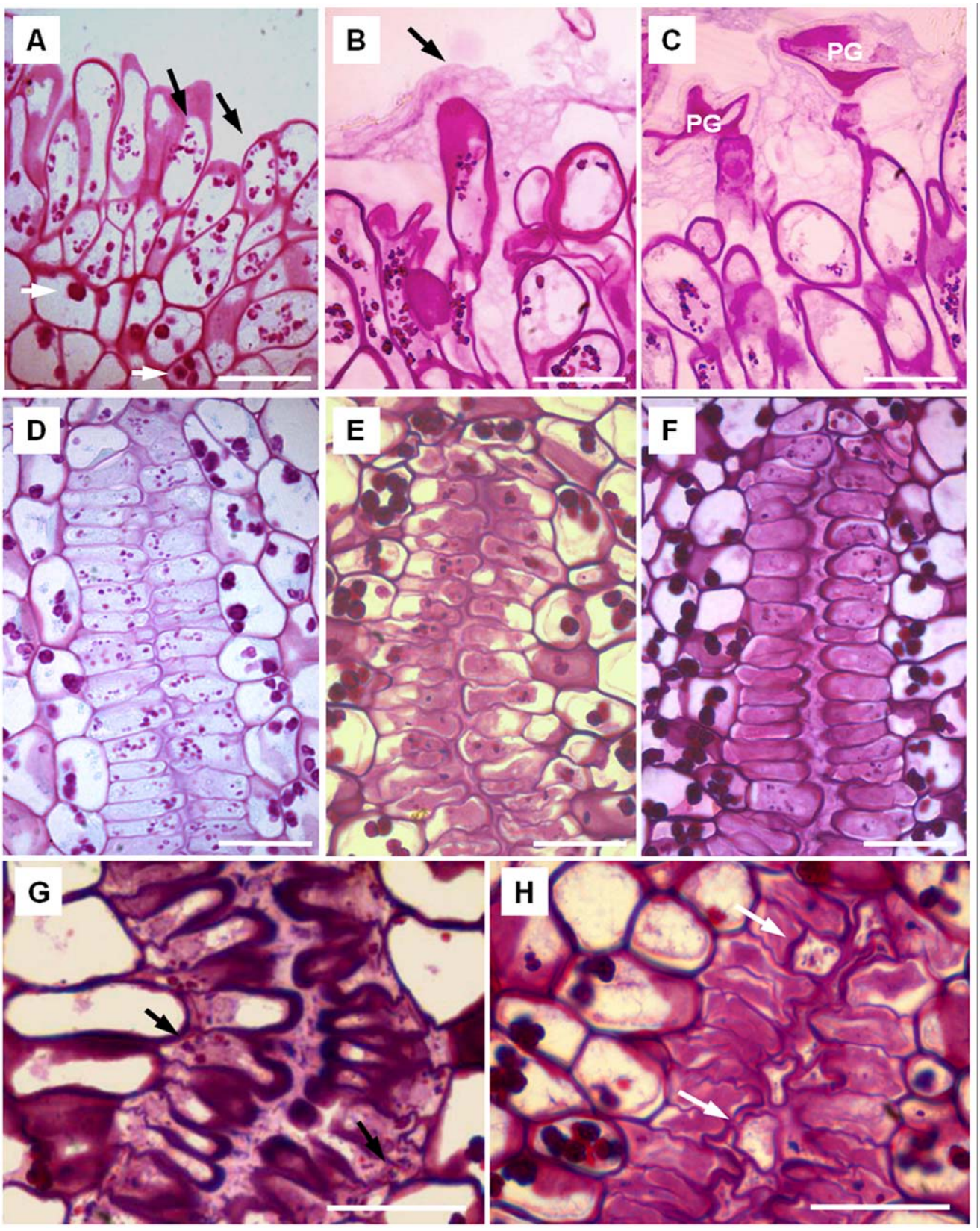

Fig. 4

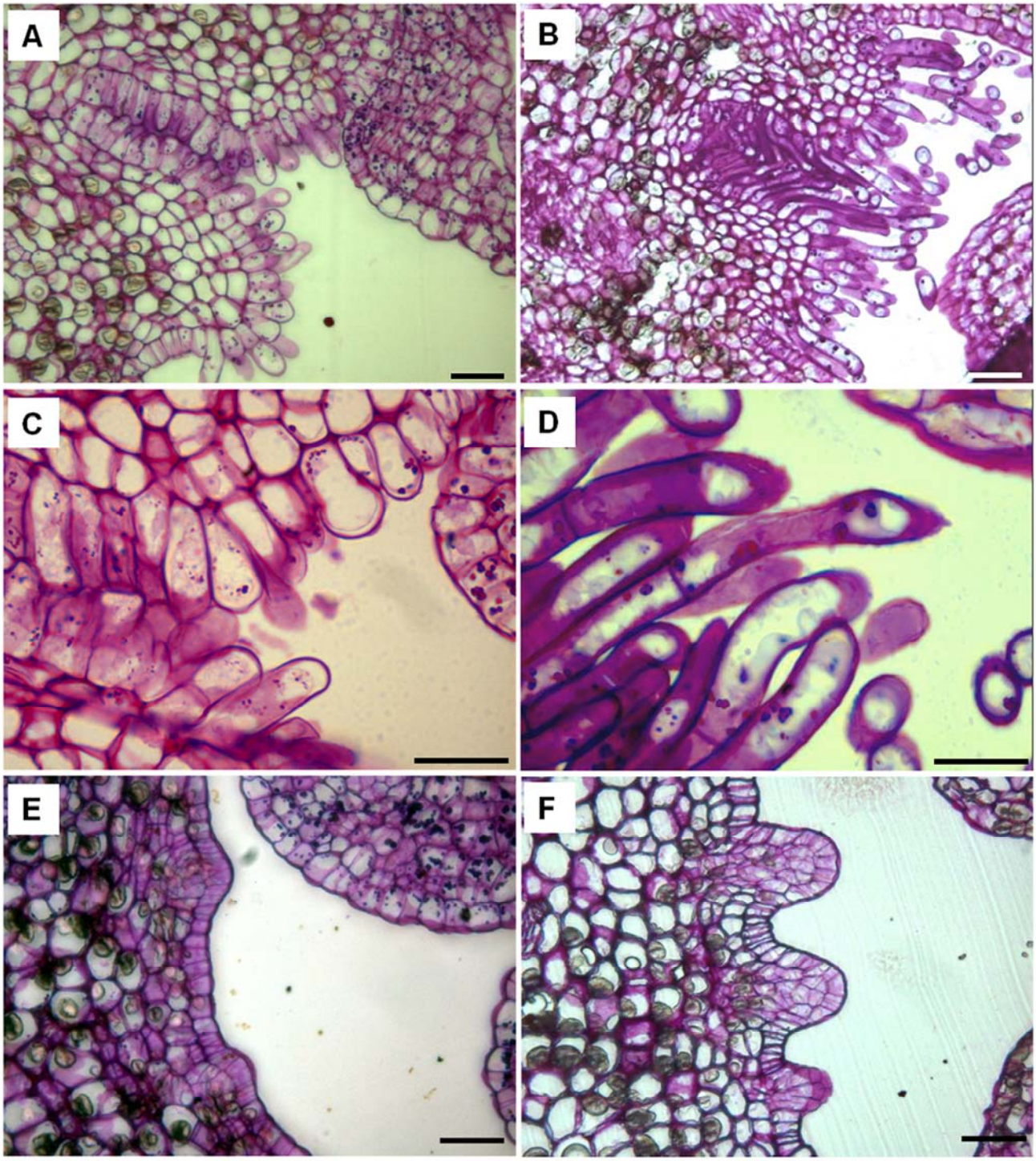

Fig. 5

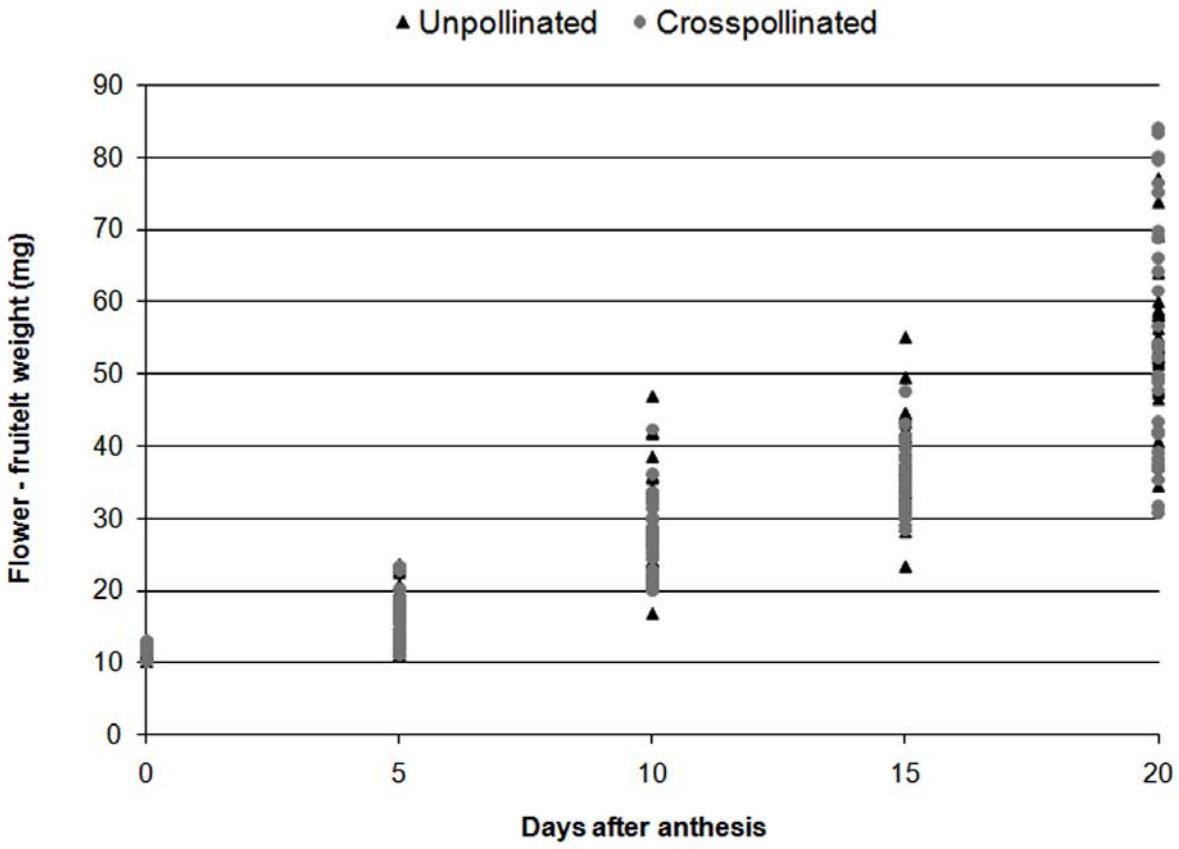
Unpollinated
Crosspollinated
Flower - fruitelt weight (mg)
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
Days after anthesis

Fig. 6